Analysis of morphological and phenological responses of diploid vs. tetraploid *Arabidopsis thaliana* to zinc toxicity

Research Thesis

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by

Logan D. Finley

The Ohio State University
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Project Advisor: Dr. Stephen M. Hovick, Department of Evolution, Ecology, and Organismal Biology

Abstract

The importance of understanding the fundamental consequences of polyploidy has been underscored by the progress made in our understanding of its prevalence in the evolutionary history of angiosperms and perhaps its acute ecological influence. Polyploidization is accompanied by a complex suite of morphological and physiological changes; in *Arabidopsis thaliana* it has previously been shown to confer enhanced tolerance to abiotic stressors such as salinity and drought. The aim of this study was to compare diploid and tetraploid *A. thaliana* individuals and identify any ploidy-linked differential responses to toxic concentrations of the micronutrient zinc. Diploid and polyploid accessions of the *A. thaliana* ecotypes Col-0 and Ler-0, 79 individuals in total, were grown hydroponically at one of five zinc concentrations ranging from sufficiency at 10 μM Zn to 200 μM. Morphological and phenological traits were compared at the onset of flowering with a combination of dose-response curve models and linear models. Significant ploidy-linked differences in trait means were detected in preliminary analyses between Col-0 accessions, primarily at low concentrations and diminishing as concentration increased, but not significantly between Ler-0 accessions. Limited sample size precluded definitive claims regarding zinc tolerance, but the asymmetry between ecotypes in their responses suggests that any adaptive changes connected to polyploidization may be less than universal.

Introduction

Polyploidization or whole-genome duplication (WGD) is well-documented in angiosperm lineages and its apparent frequency has drawn increased attention in the last two decades. The magnitude of its influence on adaptive radiation or on more acutely adaptive functional traits has become a subject of interest as our awareness of their importance has grown (Soltis et al. 2009). The persistence of polyploid lineages in nature may be attributed in part to adaptive advantages conferred by genome duplication, though the types, frequencies, and underlying mechanisms of these advantages are not well understood (Del Pozo and Ramirez-Parra, 2015). Novel or enhanced abiotic stress tolerances are particularly interesting traits that have previously been observed in polyploid individuals, made especially relevant by rising heavy metal concentrations in many urban or otherwise human-impacted environments. (Ferreira et al., 2018, Baduel et al 2018).

In the easily cultivated model plant *Arabidopsis thaliana* it has been demonstrated that polyploids show increased salinity tolerance as well as increased drought tolerance (Chao et al., 2013). Stress tolerance is an important trait observed to accompany polyploidy that appears to stem from the fact that a significant portion of the genes with altered expression in duplicated genomes are those that are responsive to various stresses like osmotic and oxidative stress (Del Pozo and Ramirez-Parra, 2014). Zinc is an essential micronutrient with a relatively low threshold for sufficiency, and as such it begins to negatively impact plant function at relatively low concentrations with symptoms including chlorosis, inhibition of root elongation, and decreased photosynthetic capacity (Ruano et al. 1988, Sagardoy et al. 2009, Van Assche and Clijsters 1986). Unlike other metals it's not thought to be regulated by exclusion at the root level, but instead by a combination of vacuolar sequestration and possibly metal-scavenging phytochelatins

(Godbold et al. 1983, Tennstedt et al. 2009). Given the potential origins and mechanisms of polyploid salt and drought tolerance such as those introduced by Del Pozo and Ramirez-Parra in 2014, I sought to investigate whether changes in the regulation of zinc homeostasis might occur in the aftermath of whole-genome duplication.

To this end I chose *A. thaliana* as the system for study because of its availability, fast growth rate, and excellent performance in indoor growth chambers. Hydroponic cultivation would serve as an inexpensive and flexible framework for experimental design as well as precise control over the composition of the growth media, and the system developed by Conn et al. in 2013 was tailored towards *Arabidopsis* and was an excellent framework for its design. I subjected diploid and tetraploid *A. thaliana* accessions to a zinc concentration spectrum from sufficiency to toxicity for a dose-response framework. By measuring response though morphological and phenological traits I aimed to test for evidence of ploidy-linked differential zinc tolerance in *A. thaliana.*

Methods

Hydroponic cultivation

I developed an inexpensive hydroponic system based on the procedures outlined in Conn et al. (2013). Black microcentrifuge tube lids were punctured and filled with germination gel medium (0.7% agar), a modified Hoagland's solution (Table 1). Seeds were placed into the punched-out openings of the lids. Discarded micropipette tip boxes were filled with liquid germination medium, and the trays from the boxes were modified to accommodate the tube lids, holding the lids upright and bathing their gel-filled bases in germination solution. In these boxes the seeds were allowed to stratify in darkness for 60 hours. I placed the seeds in a 22°C growth chamber with 8 hours of light (PPFD 150 μmol m^{-2} s^{-1} at plant level) and 16 hours of darkness.

Germinants were thinned and gradually transitioned to the full-strength basal nutrient solution (BNS) outlined in Conn et al. (2013) over a period of 3 days (Figure 1).

Food-grade polycarbonate square containers served as the final growth tanks for maturing plants; these were covered in aluminum foil to block light and limit algal growth in the nutrient solution. 21 days after their transition to the growth chamber, I transferred the germinants to their final tanks. I removed the lower 20% of a 50ml Falcon tube for each plant, keeping the tube attached to guide root growth and prevent entanglement; a hole drilled into each Falcon tube lid held one microcentrifuge lid in which the plant would remain for the rest of its life cycle. The microcentrifuge tube lids rested in the Falcon tube lids, which rested in holes cut into rafts of extruded polystyrene foam board insulation that floated on top of the nutrient solution (Figure 1). Tanks were aerated by aquarium air pumps fitted with air stones to diffuse bubbles gently; each pump aerated 10 tanks when fitted with two 5-way manifolds.

I changed the nutrient solution in the large tanks twice weekly, with time between solution changes alternating between two days and three days. All solution was prepared with DI water at 30x working concentration on the day of the solution change. Each change of the basal nutrient solution was accompanied by application of the appropriate zinc concentration to each non-control tank to achieve the target experimental concentrations.

Macronutrients	Germination Medium Working conc. (mM)	Basal Nutrient Solution Working Conc. (mM)
NH_4NO_3	0	2
KNO_3	0	3
$CaCl_2$	0.75	0.1
KCl	1	2
$Ca(NO_3)_2•4H_20$	0.25	2
$MgSO_4•7H_20$	1	2
KH_2PO_4	0.2	0.6
NaCl	0	1.5

Micronutrients	Germination Medium Working conc. (µM)	Basal Nutrient Solution Working Conc. (µM)
NaFe(III)EDTA	50	50
H_3BO_3	50	50
$MnCl_2•4H_20$	5	5
$ZnSO_4•7H_20$	10	10
$CuSO_4•5H_20$	0.5	0.5
Na_2MoO_3	0.1	0.1

Table 1 – Nutrient solution recipes. Table and recipes adapted from Conn et al. 2013.

Figure 1 – Seeds germinating in microcentrifuge tube lids (left) and an early trial block with mature plants growing in their final tanks (right).

Design

I chose a diploid and a polyploid (tetraploid) *Arabidopsis thaliana* accession for each of the ecotypes Colombia and Landsberg erecta. Seeds were sourced from the ABRC and bulked under common garden conditions. In total two diploid taxa (Col2x ('Col-0', #CS3176) and Ler2x ('Ler-0', #CS20) and two polyploid taxa (Col4x ('Col-1', #CS3151) and Ler4x ('Ler', #CS3900) were represented.

Each block consisted of five tanks varying only in zinc concentration: control/zinc sufficiency (10 μM Zn, the concentration in the BNS), 50 μM Zn, 100 μM Zn, 150μM Zn, and 200 μM Zn. A representative of each experimental taxon was randomly assigned to one of four positions in each of the final tanks, resulting in 20 plants per block. This block design was replicated a total of four times, with each block initiated one week apart to stagger the data collection giving an initial total of 80 plants; the death of one Col2x specimen at the 10μM treatment level reduced the total to 79 plants. Plants were permitted to grow until bolting, defined here as the point at which the inflorescence reached a height of 2cm above the basal rosette.

Data collection - Morphology and Phenology

When an individual bolted I recorded its rosette diameter (mm) and bolting date and removed it from its tank for transport to the lab, intact, in a 50ml centrifuge tube filled with nutrient solution from the original tank. Immediately after transport I recorded the fresh masses of three mature leaf samples along with the remaining fresh aerial mass and fresh root mass after patting the roots dry and allowing them to rest for 3 minutes. Leaf area for the 3 leaf samples was then assessed using WinFolia (Regent Instruments, Inc. Québec, Canada). Petioles were left attached to leaves for all measurements.

All biomass was then oven-dried at 60°C for at least 72 hours and dry masses were recorded for the leaf samples, remaining aerial parts, and root system. The data gathered for analysis ultimately consisted of total dry biomass (g), rosette diameter (mm), specific leaf area (mm^2mg^{-1}), root dry biomass (g), age at bolting (days), and root mass ratio.

Analyses

I conducted all analyses using R (version 3.6.2; R Core Team 2019). A linear model of total dry biomass data including all factors (ecotype, ploidy, and zinc concentration) was examined for significant three-way interaction; inferences were based on Type III sums of squares from the R package *car* (Fox and Weisberg, 2019). This analysis indicated that separate fitting of dose-response curves based on taxa was warranted, which I conducted with the *drc* package (Ritz et al. 2015). The most appropriate nonlinear model (e.g., log-logistic, Weibull, exponential) for each trait was selected based on AIC, with 4 curves separated by taxa for each trait. Total dry biomass data and dry root biomass data were best fit by a 3-parameter log-logistic model while rosette diameter and specific leaf area were matched with a 3-parameter Weibull model with lower limits set to zero. Both models are parameterized in the same manner with coefficients denoting curve steepness, upper asymptote, and estimated median effective dose (EC50 or ED50) marking the concentration expected to induce an average response halfway between the lower and upper limits. Traits assessed in this manner included rosette diameter (mm), specific leaf area ($mm^2\ mg^{-1}$) and root dry biomass (g). Specific leaf area for each individual was treated as an average of its sampled three-leaf subset. Root mass ratio (root mass (g)/total mass (g)) and age at bolting (days) were measured but not adequately characterized by any single nonlinear model, and these traits were excluded from the dose-response curve analysis.

In addition to dose-response curve fitting, each trait was assessed for significant interactions in the same manner as total biomass. Ecotype-specific linear models were analyzed, still through *car*'s ANOVA using type III sums of squares, to determine the extent of the interactions and inform post hoc Tukey tests that would allow grouping by significant difference at $\alpha=0.05$. These were conducted with the package *agricolae* (Mendiburu 2020).

Results

Total Dry Biomass

The initial ANOVA for biomass data revealed a significant interaction between factors ecotype, ploidy, and zinc concentration ($F_{1,71} = 6.22$, $p = 0.0149$). The 3-parameter log-logistic model showed a substantially higher upper biomass limit parameter *d* for Col4x (1.03g) compared to those of the other taxa; the lower bound of the 95% confidence interval for Col4x biomass exceeds the upper bounds of the intervals of all other taxa, the highest of which is 0.43g for Col2x (Table 2; Figure 2). Applying 95% CIs to ED(50) estimates for biomass likewise did not indicate significant differences in any taxa, though the estimated concentration was higher in polyploids for both ecotypes.

A subsequent ANOVA was performed for each ecotype, and these indicated a significant ploidy-concentration interaction for the Col group but not the Ler group (Table 4). Pairwise comparisons of Col biomass using Tukey's HSD test showed significantly higher mean biomass in Col4x than Col2x at 10μM, 50μM, and 100μM concentrations with polyploidy corresponding to higher biomass at all concentrations (Table 3). Ploidy-linked differences in mean biomass were not significant for Col at 150μM and 200μM, but biomass was nevertheless greater at all concentrations for polyploid Col than in its diploid counterpart. Although the Ler group lacked

significant differences in mean biomass between ploidy levels, mean biomass trended higher for polyploid than diploid Ler at all concentrations except 100µM.

Within Col4x the effects at 10µM and 50µm on total biomass, not differing from each other, were significantly different from the effects at 150µM-200µM, and there was likewise a significant difference in response at 100µM compared to 200µM. In Col2x differences in response were only detected between 50µM and 200µM. No significant concentration effect was detected in Ler2x or Ler4x but, with the exception of Ler2x achieving its maximum mean biomass at 100µM instead of 10µM or 50µM, both steadily decreased in biomass as zinc concentration increased.

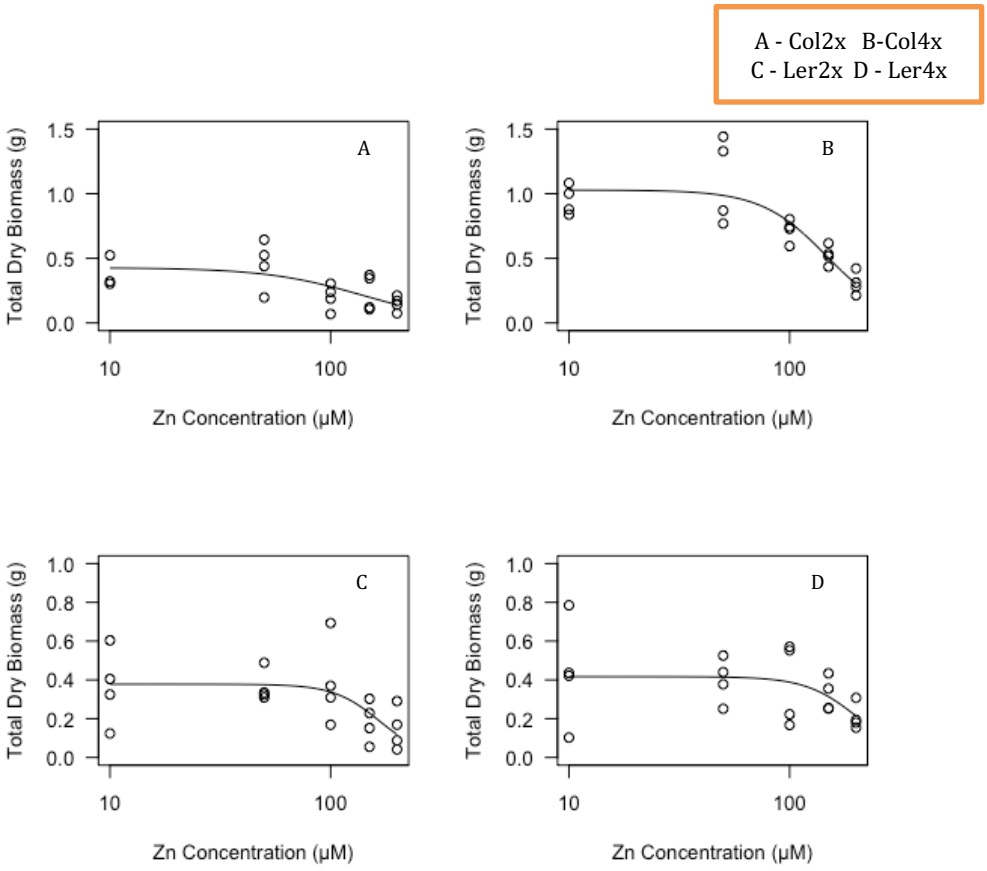

Figure 2 - Total biomass (g) vs Zinc concentration (µM), dose-response curves.

Rosette Diameter

The influence of excess zinc on rosette diameter resembled its effect on biomass. Little influence on rosette diameter was detected in the polyploid Ler group and none of its parameter estimates in the Weibull model are significant. Consequently the model was unable to resolve a true curve for the Ler4c group. The upper limit estimate *d* and its 95% confidence interval for the Col4x group (156.8mm) fell above those of the adequately modeled Col2x (102.1mm) and Ler2x (97.4mm) groups, reflecting a significantly higher mean rosette diameter at low concentrations. Also significant was the difference between ED(50) estimates for groups Col4x (102.46 ± 21.66 μM) and Ler2x (197.55 ± 38.60 μM) (Table 2; Figure 3). No significant difference was shown between the ED(50) estimates of diploid and polyploid Col, but notably the polyploid estimate is less than that of the diploid by a factor of 0.55 with only a relatively small overlap in their 95% CI's rendering the difference insignificant.

The initial linear model for rosette diameter did reveal a significant 3-way interaction between ecotype, ploidy, and concentration ($F_{1,71} = 11.77$, $p = 0.001$). Secondary models for each ecotype showed significant ploidy-concentration interaction for only the Col ecotype (Table 4). Among these, substantial pairwise differences between Col2x and Col 4x were present at 10μM and 50μM concentrations (Table 3), with rosette diameter greater for Col4x at all concentrations. Means were significantly different within Col4x between concentration clusters (10μM-50μM) and (100μM-200μM), with a significant difference detected between 100μM and 200μM concentrations as well. Within Col2x rosette diameter was only significantly different at 200μm, but still decreased with each step up in concentration.

No interaction was identified in the Ler ecotype – polyploids did not differ significantly from diploids at any concentration (Table 3; Table 4). Ler2x differed from others in its taxon only at 200μM, while Ler4x showed no significant change over the range of concentrations.

Notably, the three greatest mean rosette diameters in Ler4x occurred at 50µM, 150µM, then 100µM. Ler2x was also unusual in that its diameter at 100µM was greater than it was at 50µM, though again none of the differences at these concentrations were significant for either Ler group.

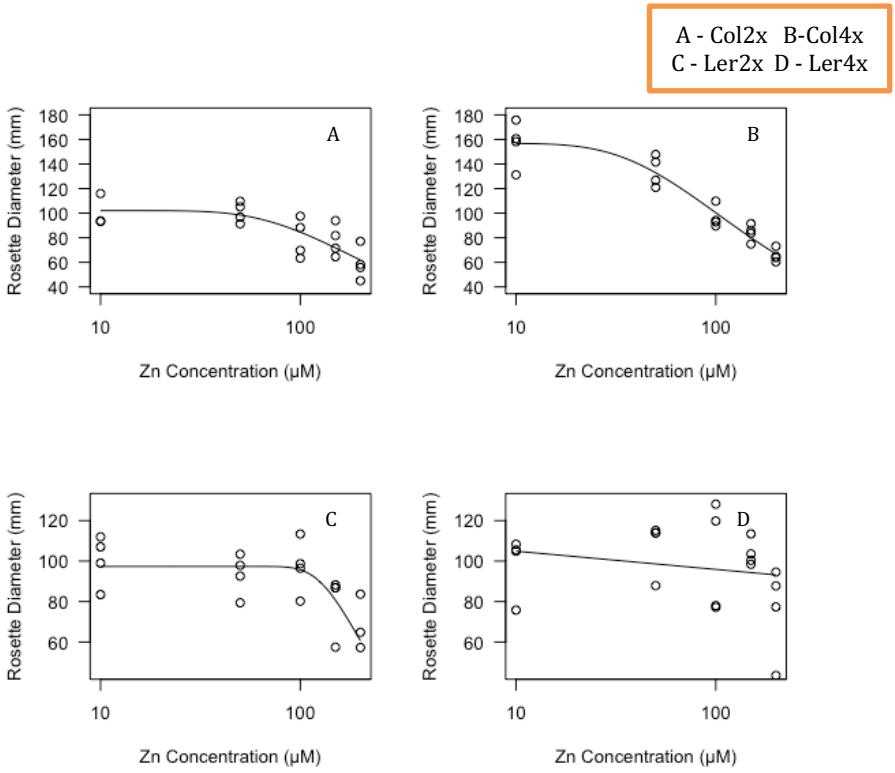

Figure 3 – Rosette Diameter (mm) vs Zinc concentration (µM), dose-response curves.

Specific Leaf Area

Generally a decrease in specific leaf area (SLA) was seen as zinc concentration increased. A reasonable fit is achieved by the Weibull model for all but the Col4x group, which has a weaker signal that did not resolve as a true response curve. Within their 95% confidence intervals the upper limit *d* does not differ significantly between any of the three well-characterized groups (Ler2x, Ler4x, Col2x). Meaningful comparison of the upper limit through the model is difficult for the poorly-characterized Col4x group, but while it falls short of assessing its significance the dose-response curve does capture a lower mean SLA for Col4x at all concentrations but 200µM. Only two groups were determined by the model to have an ED(50) significantly different from zero, Col2x and Ler4x, but their overlapping confidence intervals do not suggest a significant difference from each other (Table 2).

Assessment of the linear model for SLA showed only a significant 2-way interaction between ploidy and ecotype ($F_{1,71} = 10.57$, $p = 0.001$). Tukey's HSD revealed a significantly lower overall mean SLA for Col4x (39.94 mm^2mg^{-1}) than the other groups (Col2x=58.63, Ler2x=58.38, Ler4x=53.88 mm^2mg^{-1}) (Table 3). This extended to significant differences between Col4x and Col2x at 50µM, 100µM, and 200µM concentrations. None were found between Ler4x and Ler2x. Within taxa, differences in mean SLA by concentration were only significant for Ler4x and occurred between 50µM and 200µM for the Ler polyploids. Though it was only a significant effect in Col, the polyploid accessions of both ecotypes exhibited a lower SLA than their associated diploids.

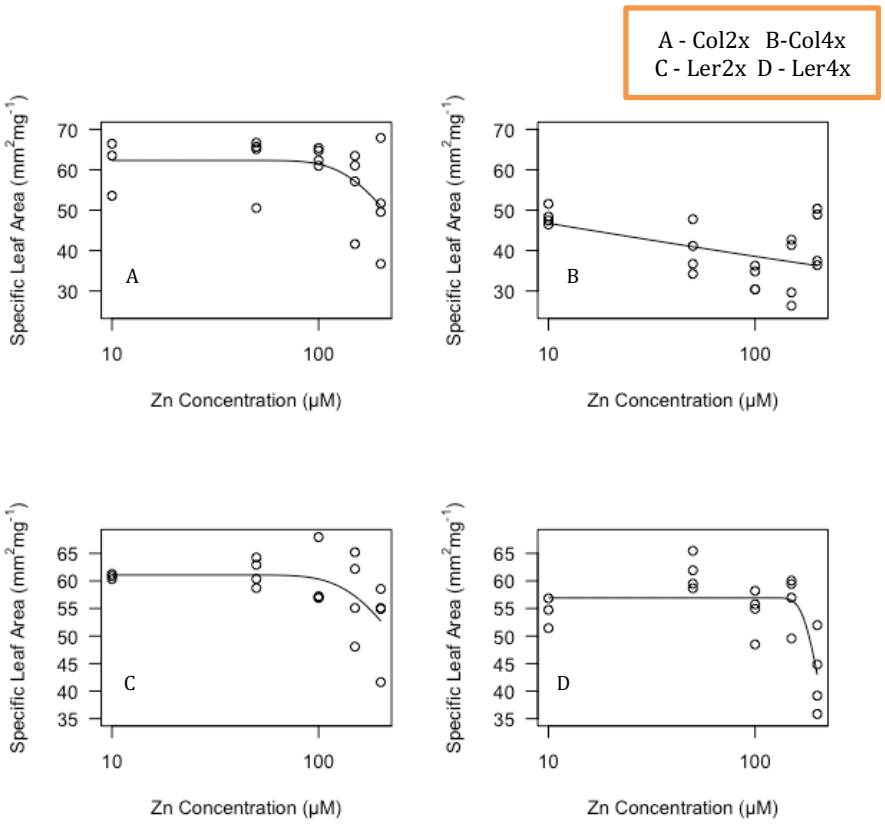

Figure 4 –Specific Leaf Area (mm^2 mg^{-1}) vs Zinc concentration (µM), dose-response curves

Root dry biomass

A marginally significant three-way interaction between ploidy, ecotype, and zinc concentration was present ($F_{1,71} = 3.80$, $p = 0.055$) as well as significant ecotype-ploidy ($F_{1,71} = 8.88$, $p = 0.004$) and ploidy-concentration ($F_{1,71} = 5.54$, $p = 0.021$) interactions. Pairwise comparisons made for the ecotype-ploidy interaction revealed significantly higher mean root mass in the Col4x group compared to the other three groups as a whole, among which there was no significant difference (Table 3). Analysis for a ploidy-concentration interaction was then performed on both ecotypes separately which showed significant interaction in Col but not in Ler (Table 4) . Pairwise comparisons of Col indicated significantly higher root mass in Col 4x than Col2x at 10µM and 50µM concentrations. No significant ploidy-linked differences were detected

between Ler4x and Ler2x, but mean root mass was higher for Ler4x at all concentrations except 100µM (Table 3).

For Col4x, a significant difference in treatment effect exists between the two concentration groups 10µM-50µM and 100µM-200µM with root biomass being higher in the former group (Table 3). Significant root mass reduction occurred in Col2x only at 200µM zinc. The maxima for Col2x and Col4x occurred at 50µM and 10µM respectively, falling by a factor of 0.26 and 0.28 to their minima at 200µM. In Ler4x the reduction in root mass was not significant at any concentration in spite of a visibly decreasing trend with increasing concentration. Ler2x, like Col2x, saw a significant decrease only at 200µM but followed the same decreasing trend.

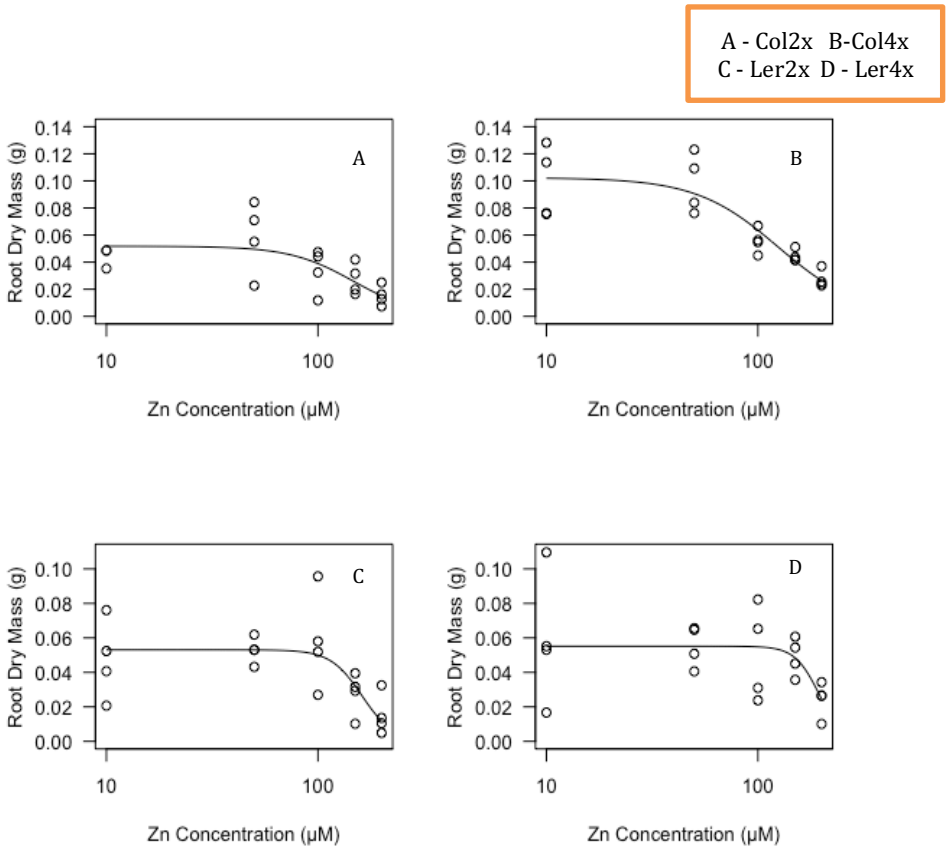

Figure 5 –Root Dry Mass (g) vs Zinc concentration (µM), dose-response curves.

Age at Bolting

An initial ANOVA indicated significance only for ploidy as a factor in bolting age ($F_{1,71}$ = 6.78, p = 0.011). However, subsequent pairwise comparisons for interaction between ploidy and concentration separated by ecotype revealed that only the Col4x group differed from its diploid pairing, and also with all other taxa (Table 3; Table 4). Col4x bolting age was significantly greater (later) than that of Col2x at 10µM, 100µM, and 150µM. However, there were no concentration-linked effects on bolting age detected in Col4x or in any other taxa. The non-significant pattern exhibited by Col4x differed from the more-or-less linear pattern of other taxa in that it resembled an inverted u-shape, increasing along with concentration until its mean decreased at 200µM.

Root mass ratio

Overall an interaction between ecotype and ploidy was detected in root mass ratio response ($F_{1,71}$ = 4.02, p = 0.048); pairwise comparisons revealed that this interaction was limited to the Col4x group, the taxon with the lowest mean root mass ratio overall of all four groups (Table 3). This interaction within Col groups consisted solely of a significant difference between Col4x and Col2x at 100µM zinc. Within taxa, mean root mass ratio was not found to vary significantly with changing concentration.

In addition to a lack of significant changes across the concentration gradient, root mass ratio trends appeared to be the farthest from monotonic of all traits measured; in Col4x mean root mas ratio increased with concentration until 100µM, at which point it began to decrease. The opposite was true for Col2x, which decreased and then increased on either side of 100µM. For Ler2x only positive change occurred with increases in concentration until root mass ratio decreased at 200µM; for Ler4x the effect of each concentration level was of the opposite sign in an increase-decrease-increase-decrease pattern.

			Col				Ler			
Response		Model term	Param. Estimate	T	p	95% Conf. Int.	Param. Estimate	T	p	95% Conf. Int.
Biomass (g)		Curve steepness	1.96	2.01	0.06	(-0.1, 4.03)	4.19	1.58	0.13	(-1.39, 9.78)
	2x	Upper lim.	0.43	6.18	< 0.001	(0.28, 0.57)	0.38	7.55	< 0.001	(0.27, 0.48)
		ED(50)	139.92	3.59	< 0.001	(57.27, 222.56)	166.11	6.05	< 0.001	(108.14, 224.09)
		Curve steepness	2.92	3.73	< 0.001	(1.27, 4.56)	3.70	0.77	0.453	(-6.46, 13.85)
	4x	Upper lim.	1.03	14.63	< 0.001	(0.88, 1.18)	0.42	6.61	< 0.001	(0.28, 0.55)
		ED(50)	147.85	10.17	< 0.001	(117.17, 178.52)	202.67	4.58	< 0.001	(109.35, 295.99)
Rosette diam. (mm)		Curve steepness	-0.92	-2.78	0.01	(-1.62, -0.22)	-2.06	-2.33	0.032	(-3.93, -0.2)
	2x	Upper lim.	102.08	15.83	< 0.001	(88.41, 115.75)	97.36	22.34	< 0.001	(88.17, 106.56)
		ED(50)	185.17	5.71	< 0.001	(116.4, 253.95)	197.55	10.80	< 0.001	(158.95, 236.15)
		Curve steepness	-0.88	-7.37	< 0.001	(-1.13, -0.63)	-0.07	-0.68	0.507	(-0.28, 0.14)
	4x	Upper lim.	156.80	28.08	< 0.001	(145.02, 168.59)	157.85	1.25	0.229	(-108.75, 424.45)
		ED(50)	102.46	9.98	< 0.001	(80.81, 124.12)	37.05	0.05	0.962	(-1568.81, 1642.91)
SLA (mm^2mg^{-1})		Curve steepness	-1.31	-1.41	0.179	(-3.29, 0.66)	-1.15	-1.07	0.301	(-3.43, 1.13)
	2x	Upper lim.	62.30	22.60	< 0.001	(56.45, 68.14)	61.07	29.98	< 0.001	(56.76, 65.39)
		ED(50)	300.53	2.62	0.019	(57.35, 543.72)	363.16	1.67	0.115	(-98.97, 825.28)
		Curve steepness	-0.12	-3.36	0.004	(-0.19, -0.04)	-4.36	-0.85	0.408	(-15.24, 6.52)
	4x	Upper lim.	88.07	-	-	-	56.91	36.03	< 0.001	(53.56, 60.26)
		ED(50)	0.95	-	-	-	216.27	10.60	< 0.001	(173.01, 259.52)
Root biomass (g)		Curve steepness	2.87	2.18	0.045	(0.08, 5.67)	5.84	2.00	0.062	(-0.33, 12.01)
	2x	Upper lim.	0.05	7.44	< 0.001	(0.04, 0.07)	0.05	9.37	< 0.001	(0.04, 0.07)
		ED(50)	147.42	5.34	< 0.001	(88.84, 205.99)	161.34	8.95	< 0.001	(123.32, 199.37)
		Curve steepness	2.24	3.94	0.001	(1.04, 3.44)	7.60	0.89	0.384	(-10.34, 25.53)
	4x	Upper lim.	0.10	13.43	< 0.001	(0.09, 0.12)	0.06	8.27	< 0.001	(0.04, 0.07)
		ED(50)	124.73	8.22	< 0.001	(92.71, 156.75)	194.22	9.64	< 0.001	(151.73, 236.72)

Table 2 – Dose-response model parameter estimates calculated using each trait's respective nonlinear model. The upper lim. estimate reflects trait means at the control Zn dose (10μM).

		Col					Ler				
	Ploidy	10 µM	50 µM	100 µM	150 µM	200 µM	10 µM	50 µM	100 µM	150 µM	200 µM
Biomass (g)	2x	0.383 (0.056) ^{cde}	0.451 (0.094) ^{cde}	0.2 (0.043) ^{de}	0.235 (0.037) ^{de}	0.15 (0.166) ^{e}	0.364 (0.042) ^{a}	0.364 (0.139) ^{a}	0.385 (0.053) ^{a}	0.184 (0.034) ^{a}	0.147 (0.058) ^{a}
	4x	0.95 (0.05) ^{ab}	1.102 (0.071) ^{a}	0.718 (0.071) ^{bc}	0.525 (0.029) ^{cd}	0.307 (0.044) ^{de}	0.436 (0.099) ^{a}	0.399 (0.111) ^{a}	0.378 (0.055) ^{a}	0.184 (0.106) ^{a}	0.209 (0.044) ^{a}
Rosette diam. (mm)	2x	100.99 (6.398) ^{b}	100.74 (6.67) ^{b}	79.7 (4.493) ^{bc}	77.96 (3.466) ^{bc}	59.038 (6.242) ^{c}	100.634 (4.566) ^{abc}	97.036 (3.353) ^{bcd}	88.471 (6.636) ^{cde}	78.91 (4.376) ^{cde}	60.035 (6.554) ^{e}
	4x	156.46 (7.476) ^{a}	134.305 (7.95) ^{a}	96.58 (4.112) ^{b}	84.025 (9.276) ^{bc}	65.575 (2.697) ^{c}	127.545 (4.372) ^{a}	121.021 (5.856)^{ab}	98.681 (5.336) ^{abcd}	93.99 (12.264) ^{bcd}	70.688 (5.73) ^{de}
SLA (mm^2/mg)	2x	61.171 (4.91) ^{ab}	61.997 (1.029) ^{a}	63.35 (3.896) ^{a}	55.81 (6.398) ^{abc}	51.463 (3.836) ^{abcd}	60.824 (3.83) ^{a}	61.561 (0.274) ^{a}	59.769 (3.734) ^{a}	57.64 (1.247) ^{a}	52.544 (3.53) ^{aba}
	4x	48.465 (1.109) ^{abcde}	39.966 (4.113) ^{cde}	32.979 (2.966) ^{e}	35.006 (3.684) ^{de}	43.289 (1.513) ^{bcde}	54.345 (2.409) ^{ab}	61.391 (2.73) ^{a}	54.348 (2.076) ^{ab}	56.502 (1.578) $_{a}$	42.962 (1.526) ^{b}
Root biomass (g)	2x	0.044 (0.013) ^{bc}	0.058 (0.006) ^{b}	0.034 (0.004) ^{bc}	0.027 (0.002) ^{bc}	0.015 (0.011) ^{c}	0.047 (0.014) ^{a}	0.053 (0.006) ^{a}	0.058 (0.014) ^{a}	0.028 (0.005) ^{a}	0.015 (0.006) ^{a}
	4x	0.098 (0.004) ^{a}	0.098 (0.008) ^{a}	0.056 (0.004) ^{b}	0.045 (0.013) ^{bc}	0.027 (0.003) ^{bc}	0.059 (0.012) ^{a}	0.055 (0.006) ^{a}	0.051 (0.004) ^{a}	0.049 (0.019) ^{a}	0.024 (0.005) ^{a}
Age at bolting (days)	2x	65 (2.394) ^{c}	70.25 (1.031) ^{bc}	65 (4.07) ^{c}	68.5 (2.872) ^{bc}	69 (3.351) ^{bc}	62.75 (1.702) ^{a}	69 (3.198) ^{a}	70.25 (2.75) ^{a}	64.5 (2.658) ^{a}	67.75 (1.581) ^{a}
	4x	71.25 (2.81) ^{bc}	81.25 (3.227) ^{ab}	87.25 (2.887) ^{a}	88.5 (2.646) ^{a}	76.25 (1.472) ^{abc}	65.5 (3.082) ^{a}	67.25 (4.975) ^{a}	72 (5.543) ^{a}	64.75 (1.601) ^{a}	69.75 (5.809) ^{a}
RMR	2x	0.126 (0.006) ^{abc}	0.127 (0.017) ^{abc}	0.171 (0.03) ^{a}	0.131 (0.006) ^{ab}	0.101 (0.008) ^{bc}	0.137 (0.006) ^{ab}	0.147 (0.009) ^{ab}	0.157 (0.011) ^{ab}	0.162 (0.01) ^{a}	0.108 (0.008) ^{b}
	4x	0.102 (0.004) ^{bc}	0.091 (0.004) ^{bc}	0.078 (0.004) ^{c}	0.085 (0.007) ^{bc}	0.091 (0.004) ^{bc}	0.139 (0.009) ^{ab}	0.142 (0.009) ^{ab}	0.136 (0.009) ^{ab}	0.153 (0.016) ^{ab}	0.116 (0.018) ^{ab}

Table 3 – Trait means grouped by pairwise comparisons within taxa. Superscript groupings denote significantly different means within a single ecotype.

		Col		Ler	
Response	Model term	F	p	F	p
Biomass (g)	Ploidy	49.92	< 0.001	16.58	< 0.001
	Zn conc.	6.76	0.013	7.09	0.009
	Ploidy × Zn	9.93	0.003	2.48	0.119
Rosette diameter (mm)	Ploidy	44.72	< 0.001	12.66	0.001
	Zn conc.	27.83	< 0.001	24.97	< 0.001
	Ploidy × Zn	18.24	< 0.001	2.02	0.159
SLA (mm^2/mg)	Ploidy	21.15	< 0.001	0.40	0.532
	Zn conc.	4.30	0.045	4.95	0.033
	Ploidy × Zn	0.54	0.469	0.50	0.484
Root biomass (g)	Ploidy	26.00	< 0.001	10.31	0.002
	Zn conc.	12.03	0.001	16.23	< 0.001
	Ploidy × Zn	7.15	0.011	1.69	0.197
Age at bolting (days)	Ploidy	6.94	0.012	0.04	0.844
	Zn conc.	0.20	0.660	0.18	0.671
	Ploidy × Zn	0.44	0.509	0.00	0.956
RMR	Ploidy	10.93	0.002	0.24	0.627
	Zn conc.	1.91	0.176	1.34	0.255
	Ploidy × Zn	0.29	0.595	0.03	0.857

Table 4 – Ecotype-specific linear models, tests for significant interactions

Discussion

From a morphological standpoint the differences between Col4x and Col2x are striking. By the time bolting occurred, the results indicate that the polyploid Col4x accession was more massive both overall and with respect to roots alone, larger in diameter, and older than the diploid Col2x irrespective of statistical significance. Because of this consistency and the overall strength of the signals in this small dataset, it seems likely that greater statistical power from a larger sample size would preserve and strengthen the tentative relationships illustrated here between Col accessions, certainly for lower zinc concentrations. Less certain, though, is whether meaningful differences would emerge between them at the highest zinc concentrations tested here. At 200μM the only significantly differing trait detected between Col accessions was specific leaf area. This is not necessarily remarkable given the fact that SLA was lower for Col4x than Col2x at all concentrations, albeit not always significantly.

The dramatic shrinking of the distance between Col2x and 4x trait values could be interpreted in a number of ways: It may suggest that the effects of zinc toxicity are relatively proportional to the traits measured here such as biomass or root biomass or an increased susceptibility of polyploid Col to zinc toxicity, or that there is a degree of adaptive plasticity in Col4x that is not shared by Col2x that might correspond to increased fitness or survival after metal accumulation. Insight can perhaps be gained by more closely examining the directionality of changes in root mass ratio, though it should first be noted that concentration-linked changes in root mass ratio were not statistically significant in any of the four taxa. The greatest separation of mean root mass between any taxa occurred between Col2x and Col4x at 100μM; compared to their means at the previous concentration, 50μM, Col2x increased and Col4x decreased to create this distance. Because the change in total biomass and root biomass, as well as aboveground biomass (data not shown) was negative for both groups between 50μM and 100μM and occurred

nearly in parallel, an increase in mean root ratio could only have been caused by aerial (and therefore overall) biomass decreasing more quickly than root biomass. The opposite effect, a decreasing root mass ratio as observed in Col4x, would arise from a root mass decrease that outpaced that of aerial mass. Inhibition of root growth and elongation is a hallmark of zinc toxicity (Godbold et al. 1983, Ruano et al. 1988). It's possible, then, that the pattern observed in mean root mass hints at susceptibility to zinc toxicity in polyploid Col relative to the diploid, though this may be indistinguishable from plasticity within the scope of this experiment. If roots are disproportionately impacted, the root length and diameter data that were collected as part of this study may prove useful in future analyses for quantifying overall zinc effects. Importantly, in addition to the fact that this notion of reduced Col4x zinc tolerance is based on data that were not deemed statistically significant, the observations made in this study fall far short of refuting the opposite claim: that polyploid Col demonstrates an increased ability to tolerate zinc by virtue of its maintenance of consistently different means from Col2x at high concentrations.

The low SLA overall of Col4x compared to all other taxa is interesting on two levels, the first of which being the general association of lower SLA with slower growth strategies (Lambers and Poorter 2005). This may be reflected in the significantly later flowering times observed only among Col4x individuals. Second, there is an inverse relationship between SLA and persistence in stressful conditions, though these are most typically discussed in the context of water or nutrient scarcity or richness rather than toxicity; consistent reductions in SLA in the grass *Brachypodium genuense* for example were found in populations that had adapted to stressful "low productive conditions" when compared to those growing in more productive conditions (Tardella et al. 2017). These potential impacts are complicated somewhat by a variable that would have been obscured by my methodology: differences in petiolar length between taxa. Petiolar tissue was included in the measurement of both leaf area and leaf mass,

likely contributing more to increases in mass than in area. The petioles of Col4x were generally longer than in other taxa, which may account for its lower SLA.

The morphological picture painted by the Ler ecotypes is less clear than the Col ecotypes. The pairwise comparisons made between them could not discern significant ploidy-linked differences in any of the traits measured. Notably, but never significantly, higher values were observed in Ler4x than 2x for total biomass, root biomass, SLA, and rosette diameter, with the difference between their means increasing slightly towards the highest concentrations of zinc. Determining whether or not this difference indicates truly diverging means would certainly require larger sample sizes given the variance seen in these data.

The most salient difference between the Col and Ler taxa was the obvious divergence of most means between Col groups that made the Ler groups practically indistinguishable by comparison. Among the measured traits, Col taxa are very obviously phenotypically distinct from each other. There are no significantly different means that suggest the same among Ler taxa even though they reproduce the Col patterns to an extent, albeit on a much smaller scale. Another point of difference between the ecotypes is the fact that the Ler2x biomass and root biomass maxima occurred at 100μM, differing from Col2x's maxima for both traits at 50μM. Col4x total and root biomass maxima occurred at 50μM and 10μM respectively, while Ler4x peaked at 10μM for both traits. These differences do highlight a pattern shared across ecotypes: traits concerning biomass had maximum means centered toward more intermediate concentrations in diploids and towards lower concentrations for polyploids. But ultimately the two groups differ conspicuously in the extent of their ploidy-linked morphological changes, suggesting that the mechanism for these changes yields consequences that are either wildly unpredictable or simply not universal, possibly even differing among every instance of polyploidization.

In the case of this study it is easiest and perhaps even most useful to make comparisons with the linear models rather than the nonlinear dose-response curves. This can be attributed in large part to the experiment's small sample sizes and high variance. In their current form the dose-response models have limited resolution that would be greatly improved by a larger data set, particularly with more data collected at higher zinc concentrations than those used here. The lower limit of the models has been set to zero, which doesn't necessarily reflect the biological reality of continuous variables such as biomass. A more realistic lower limit might be set by experimentally determining the highest viable concentrations, perhaps those approaching the LD(50). Solidifying the endpoints of the curves would greatly improve steepness parameter estimates of the models and the quality of the ED(50) estimates, increasing the utility of ED(50) as a measure of zinc tolerance overall.

The pairwise comparisons made in this analysis are useful in the sense that they are straightforward, but their statistical power is low enough that the likelihood of overlooked significance is high. The presence of some signals in spite of this fact further suggests that this may be the case. Additionally, degrees of freedom were lost in the linear models leading to the pairwise comparisons by discretizing zinc concentration as a categorical variable, further reducing their statistical power.

Conclusion

The results of this study raise questions about the likelihood of universality in polyploidy's physiological and morphological consequences. These findings suggest that its effects may have the potential to vary in magnitude or even their presence at all, possibly varying by lineage. The nature of *A. thaliana* seed stock is another factor to consider here given the intentional homogeneity of the lineages produced by colchicine-induced polyploidy. Low-power

statistical tests did not detect significant differences between ploidy levels of the Ler ecotype at any concentration, but ostensibly insignificant differences were observable and largely unidirectional. Clear differences were exhibited between the Col representatives demonstrating ploidy's influence, but the question of differential zinc tolerance remains unanswered thus far. Future research should involve multiple lineages as well as analyses that can directly assess the presence and influence of zinc such as tissue assays and photosynthetic metrics. It will continue to be important to unravel the nature of polyploidization's influence on evolution and ecology from a functional standpoint, regardless of whether its mystery stems ultimately from complexity or chaos.

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